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PRINCIPAL INVESTIGATOR: Caroline Evangelista Harbeson
Steven A. Rosenzweig, Ph.D.

CONTRACTING ORGANIZATION: Medical University of South Carolina
Charleston, SC 29425

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6. AUTHOR(S)
Caroline Evangelista Harbeson
Steven A. Rosenzweig, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Medical University of South Carolina
Charleston, SC 29425

E-Mail: evangelc@musc.edu

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The insulin-like growth factor (IGF) system has been shown to play an important role in the development and progression of breast cancer. IGF-1 binds with high affinity to a family of proteins called the IGF binding proteins (IGFBPs), which act as natural inhibitors of IGF-1 through sequestration from the IGF-1 receptor. The purpose of this proposal is the development of IGF antagonists based on the structure of the IGFBPs. Knowledge of the mechanism of IGFBP action is currently unclear, therefore we have taken a photoaffinity labeling approach to identify the IGF binding domains on the IGFBPs. Previous data collected in the lab indicate that the carboxy terminus of IGFBP-2 is involved in high affinity binding to IGF-1; these experiments are being repeated with IGFBP-3 to extend these findings. Current photoaffinity labeling data indicate that the carboxy terminus of the IGFBP-3 protein may be involved in IGF-1 binding. This information will be used in the rational design of therapeutic IGF antagonists for use in the treatment of breast and other cancers.

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Caroline Evangelista Harbeson

Introduction

The insulin-like growth factor (IGF) system has been shown to play a key role in the progression of breast cancer via activation of the IGF-1 receptor by the ligands IGF-1 and IGF-2. The IGFs also bind with high affinity to a family of proteins called the IGF binding proteins (IGFBPs), which serve as natural IGF inhibitors through sequestration of IGF-1 from its receptor. Currently no therapeutic IGF antagonists exist. The purpose of this project is the development of novel therapeutic IGF antagonists based on the structure and function of the IGFBPs. A photoaffinity labeling approach is being used to identify the IGF binding domains on the IGFBPs. This information will be used in the rational design of therapeutic IGF-1 antagonists for use in the treatment of breast cancer.

Body

Specific Aim 1 as outlined in the approved Statement of Work involves expression of IGFBP-3 and IGFBP-5 in bacterial and eukaryotic (mammalian) expression systems. The cDNA for IGFBP-5 has been successfully cloned into an inducible bacterial expression system. The protein is produced by the bacteria, which are then lysed to collect the cell contents that contain the IGFBP. The bacteria makes the IGFBP in an unfolded state that must be treated with reducing agents (such as urea) and then carefully refolded to obtain the native, disulfide-bond containing conformation of the protein. The current yield from an average preparation from bacteria (500 ml culture) is low, however, in microgram quantities instead of the expected milligram amounts even in the presence of the inducing agent isopropyl β-D-1-thiogalactopyranoside (IPTG). The purification procedure is currently being optimized to include clones of bacteria that have demonstrated higher expression of the binding protein.

The cDNA for rhIGFBP-3 has been successfully transfected into Chinese hamster ovary (CHO) cells for mammalian expression, which secrete the protein in its native folded and glycosylated form. Clones that express IGFBP-3 are currently being isolated and analyzed to determine protein yield. Several clones were previously isolated that expressed rhIGFBP-3 but these cells were lost due to contamination problems in the tissue culture facility. New clones are currently being analyzed and will be used to collect significant amounts of rhIGFBP-3. This expression system involves the use of methotrexate to induce amplification of the rhIGFBP-3 DNA incorporated into the mammalian chromosome. Increasing concentrations of methotrexate in the feeding media are being used to optimize the amount of protein being produced by the CHO cells. This has been a slow process because it takes time for the cells to adapt to the presence of methotrexate in their media, which initially kills a large percentage of the cells. A bacterial expression system has not been developed for rhIGFBP-3 as yet, and rhIGFBP-5 cDNA has not yet been transfected into CHO cells for mammalian expression. In the meantime, we have been purchasing recombinant IGFBP-3 in order to perform our photoaffinity labeling experiments. This form of IGFBP-3 has been expressed in bacteria and is therefore non-glycosylated. It has been shown in the literature that non-glycosylated IGFBP-3 has the same affinity for IGF-1 as native rhIGFBP-3. Bacterially expressed IGFBP-5 is not currently commercially available; therefore the experiments outlined using IGFBP-5 have not been performed. Expression of rhIGFBP-3 and -5 is currently being performed by

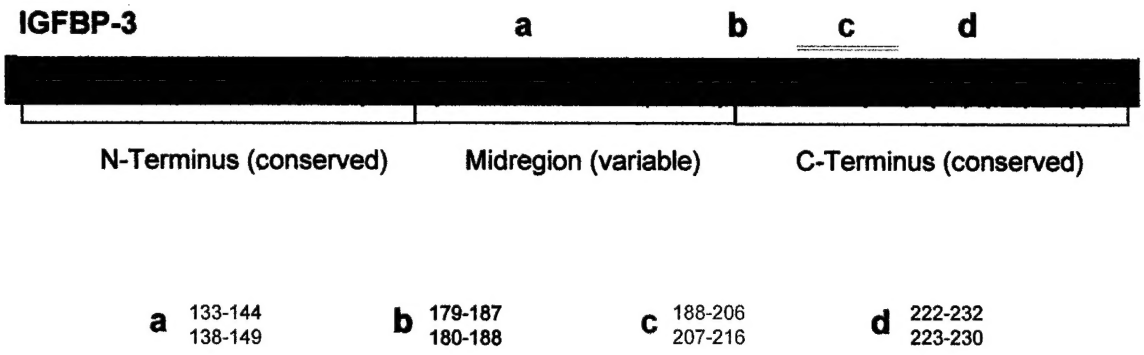
the protein expression facility in the Hollings Cancer Center, which is run within our department. This is a new facility that was recently set up, which is part of the reason why this aspect of Specific Aim 1 has not yet been completed.

Photoaffinity labeling experiments have been carried out using IGF-1 derivatized at the Gly-1 residue using 4-azidobenzoyl-N-hydroxysuccinimide ester (HSAB). This photoprobe, referred to as abGly1-IGF1, has been crosslinked to IGFBP-3 as outlined in Specific Aim 1. Mass spectrometric analysis of the reduced and alkylated and trypsin digested photolabeled complex reveal photoincorporation of the IGF photoprobe into several sites within the midregion and carboxy terminus of the IGFBP-3 protein (Figure 1). The sites identified by mass spectrometric analysis are distinct from each other in the primary sequence of IGFBP-3, but homology modeling with the cathepsin binding site on thyroglobulin suggest that disulfide bonds in the region identified may place the sites within close proximity to each other in three-dimensional space. These findings suggest that the binding mechanism between IGFBP-3 and IGF-1 is a dynamic interaction that leads to high affinity binding of the proteins. These results corroborate with other data in our laboratory that suggest that the region of IGFBP-3 that contain the above sites of crosslinking are necessary for IGF-1 binding. We can conclude that the binding of IGF-1 to IGFBP-3 is distinct from that to IGFBP-2, despite the high degree of homology that exists between IGFBP-3 and -2.

Specific Aim 2 of the Statement of Work proposed identifying a potential site of interaction between the IGFBPs and the IGF-1 receptor binding domain on IGF-1 using a crosslinking reagent (HSAB) attached to the Lys-27 residue of IGF-1, a residue that is located near the IGF-1 receptor binding domain. This photoprobe is referred to as abLys27-IGF1. This photoprobe has been used to photolabel IGFBP-2 to identify sites of interaction between IGFBP-2 and the IGF-1R binding domain on IGFBP-2. The rhIGFBP-2 being used in these experiments is collected from a mammalian expression system, which has been used in our lab for several years. Similar to the system being optimized for IGFBP-3, CHO cells expressing rhIGFBP-2 secrete the protein into the media, which is then collected and rhIGFBP-2 is purified from the media using IGF affinity chromatography. Preliminary mass spectrometric analysis of the reduced and alkylated and trypsin digested photolabeled complex failed to confirm a site of incorporation of the abLys27-IGF1 photoprobe into IGFBP-2. The lack of results in this experiment could potentially be due to the fact that the binding interaction between the IGF-1R binding domain on IGF-1 and IGFBP-2 may be of significantly lower affinity than the IGFBP-binding domain on IGF-1, and therefore increased amounts of protein must be used in order to obtain enough crosslinked protein to be adequately analyzed by mass spectrometric methods. These studies are therefore being repeated as outlined in this proposal, in addition to using an in-gel tryptic digest approach, in which the reduced and alkylated photolabeled complex is run on SDS-PAGE gel, the gel is stained with copper chloride, the bands of appropriate molecular weight corresponding to the cross-linked proteins are cut out of the gel and trypsin is again used to generate enzymatic fragments whose molecular weight can be identified through mass spectrometric methods as outlined in the proposal. These studies will help determine if the IGFBPs interact with the receptor-binding domain on IGF-1, thus suggesting a mechanism by which the IGFBPs inhibit IGF-1 access to its receptor.

Specific Aim 3 as outlined in the approved Statement of Work has not yet been addressed.

Figure 1. Sites of photoincorporation by abGly1-IGF1 into IGFBP-3 and the corresponding residues.



Key Research Accomplishments

- IGFBP-5 has been expressed in an inducible bacterial expression system (*E. coli*) and the purification procedure is currently being optimized.
- IGFBP-3 has been expressed in a mammalian expression system (CHO cells) and the system is currently being optimized.
- Photoaffinity labeling of IGFBP-3 using abGly1-IGF1 identified the midregion and carboxyl terminus of IGFBP-3 as being involved in IGF binding.
- Photoaffinity labeling of IGFBP-2 using abLys27-IGF1 indicated that crosslinking occurred, however the site of contact has not yet been identified by mass spectrometric analysis.

Reportable Outcomes

1. Work described in this report has been presented in poster format at the 84th annual meeting of the Endocrine Society, of which I am a fellow/student member. The data was presented in poster format on June 20, 2002 in San Francisco, CA.
2. Work described in this report has been presented in oral format at MUSC's annual Student Research Day, on November 1, 2002. I received 2nd place in my session for PhD students and recognition from the department and school for this honor.
3. rhIGFBP-3 has been expressed in a mammalian expression system using Chinese hamster ovary cells. The system is currently being adjusted for optimal expression of IGFBP-3 protein.
4. rhIGFBP-5 has been transfected into a bacterial expression system. This system is also currently being adjusted for optimal expression of IGFBP-5 protein.

Conclusions

In conclusion, several points of Specific Aim 1 of the proposed Statement of Work have been completed. IGFBP-3 has been expressed in a mammalian system, and IGFBP-5 has been expressed in a bacterial system, and both systems are currently being adjusted to promote optimal protein yield. Current photoaffinity labeling experiments with IGFBP-3 have identified the midregion and carboxyl terminus of the protein as being involved in IGF-1 binding. This is an important finding because a number of studies in the literature have implicated a hydrophobic region within the amino terminus of IGFBP-3 and -5 as containing a necessary binding site for IGF-1. While this data does not negate the importance of either of these regions of the binding proteins, it suggests that the midregion of the IGFBPs may also play an important role in IGF-1 binding. These data also suggest that the binding of IGF-1 with IGFBP-3 is distinct from its binding to IGFBP-2, suggesting that while the binding proteins contain a high degree of homology, they do not necessarily bind to IGF-1 using the same mechanism. This finding is different from what we found with IGFBP-2, so we can conclude that the interactions of the IGFBPs are unique to each protein in the sense that each binding protein interacts with IGF-1 using a different mechanism. In addition, photoaffinity labeling of IGFBP-2 using abLys27-IGF1 to identify regions of IGFBP-2 that interact with the IGF-1R binding domain on IGF-1 is currently being performed. The data described in this report corroborate other data in our laboratory that suggest the carboxyl terminus of IGFBP-3 is important to IGF-1 binding. The information gained from these studies will aid in the future development of novel IGF antagonists based on the structure and function of the IGFBPs.